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Mechanisms of Action of Low Molecular Weight Toxins in the Cardiovascular System

Annual and Final Report

W. T. Woods, Jr.

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This contract (DAMD17-86-C-6075) was devoted to investigation of mechanisms by which T-2 toxin, Roridin A, and other low molecular weight toxins exert toxic effects on the mammalian cardiovascular system. In year 02 we were granted permission by USAMRDC, to complete work on trichothecenes and phase in experiments to determine mechanisms of action of other low molecular weight toxins in cardiac cells. The majority of the second year was occupied by analysis of data obtained in earlier experiments. With respect to trichothecene toxicity, our data confirmed the hypothesis that cardiovascular complications mainly result from imbalance within the autonomic nervous system; the imbalance is caused by direct effects on autonomic neurons. With respect to the toxins known as tetrodotoxin (TTX), batrachatoxin (BTX), and veratridine, each of which binds to sodium channels in excitable cell membranes, our data suggest that BTX and veratridine can promote opening of cardiac sodium channels

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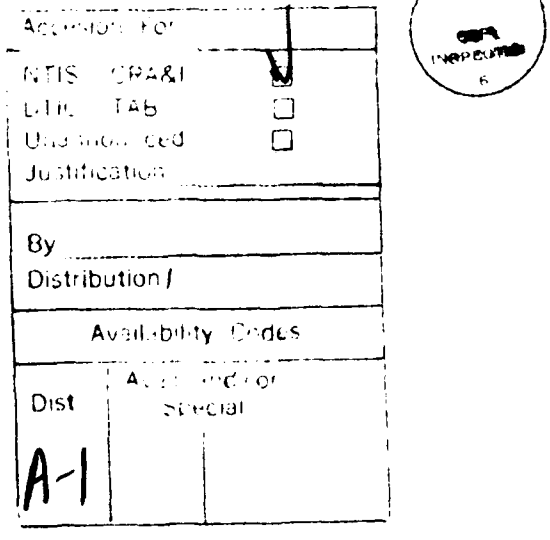
and that TTX promotes their closure. These studies demonstrated antagonistic effects and suggested how they might be applied to alter activities of different kinds of cardiac cells. In future studies we hope to elucidate mechanisms of action of saxitoxin and blue-green algae toxins in cardiac muscle and conduction system cells.



FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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TABLE OF CONTENTS

INTRODUCTION

The first contract year established how trichothecenes injected intravenously after performance of the cardiovascular system. Because of budget curtailment, the second contract year was devoted to data analysis generated during experiments on mycotoxins and other toxins. Following this section, a bibliography of publications supported by this contract is included.

MYCOTOXIN MECHANISMS OF ACTION

A meeting held at USAMRIID in January 1986 concluded that three studies were viewed as high priority. First, the question of whether or not high pulmonary vascular resistance contributes to T-2 toxicosis was to be answered with whole animal studies. Second, the question of whether effects of T-2 and roridin-A on the cardiac conduction system are mediated via the the cell membrane or via intercellular communication was to be answered with single cell studies. And third, the question of whether or not effects of T-2 are mediated through the ATP-sensitive K^+ channel was to be answered with patch clamp studies. These particular studies were be substantially complete by October 1, 1986.

Past studies (1,4,5,8,9) and recent ones in this and other laboratories (7,10,12,15-18,21-23,25) have suggested that trichothecenes can have lethal effects on mammalian cardiovascular and nervous systems. One such effect, sudden death due to abnormal heart rhythm, could result from dysfunction of cardiac cells or from damage to cardiac nerves. We have observed that T-2, some of its metabolites, and certain macrocyclic

trichothecenes can alter electrical activity in cardiac cells and they also interfere with autonomic neural control of the heart and circulation.

These studies were performed in rats, swine, and dogs. Electrocardiograms (ECG's) and arterial and venous pressures were recorded to continuously monitor cardiovascular responses to intravenous trichothecenes. To elucidate mechanisms for the previously observed changes in heart rate, cardiac pacemaker regions (sinus nodes) were excised and perfused in vitro where electrical activity in individual cells could be studied with microelectrodes (29). Whenever trichothecenes were found to alter transmembrane potentials or ionic currents, single isolated atrial or ventricular (24) cells were studied with the technique known as patch clamp (voltage clamp of a patch of membrane) in which conductance of certain membrane channels was measured (33,34). Thus, we investigated which transmembrane currents are affected.

Both the low vascular tone and reduced cardiac output implicate a disturbance in autonomic neural activity. Hence we tested the hypothesis that trichothecenes block myoneural transmission and/or produce cerebral ischemia (via pulmonary hypertension or low cerebral blood flow).

Project Title: Effects of Intravenous T-2 and Roridin A on the Canine Cardiovascular System.

This was a study of trichothecene effects in whole anesthetized animals and this year's results confirmed our previous ones that have been published. Extensions of these studies to the level of the cardiac cell membrane were accomplished by applying the techniques described in this report.

Project Title: Trichothecene-induced Action Potential Changes in Canine Atrial Working Myocardium.

The nature of changes produced by T-2, roridin A, and other trichothecenes in cardiac working muscle cells (atrial and ventricular) do not suggest that they are likely substrates for trichothecene-induced arrhythmias. Thus, membrane conductance mechanisms in other cardiac cells sich as the sinus node pacemaker and A-V node were studied.

Project Title: Electrophysiologic Effects of Trichothecenes on Canine Sinus Node Pacemaker Cells.

Although the predominant responses to intravenous T-2 and roridin A were hypotension and reflex tachycardia, simultaneous direct effects on sinus node pacemaker cells developed progressively. Sinus node firing rate became slower as pacemaker cell maximum diastolic potential became more negative, and this progressed to sino-atrial block or sinus arrest. If at the same time the A-V junction substitute pacemaker were to become suppressed, this would be a potentially lethal electrophysiologic event. Membrane mechanisms for pacemaker suppression were investigated in isolated cells.

Project Title: Effects of Trichothecenes on Cell Membrane Ion Conductance.

We have recently adapted a cell dispersion technique to our isolated, perfused canine atrial preparation and we find that a high yield of atrial cells can be reproducibly harvested from hearts of any age. Critical points in this procedure appear to be 45 min. arterial perfusion with 0.1% collagenase, $[Ca^{++}]$ = 0.03 millimole/liter or less, and maintenance of normal arterial pressure and temperature.

The collagenase-treated tissue is minced with scissors and mildly agitated in 10 ml. Ca^{++} -free perfusate for 10 min. It is then filtered

through a nylon mesh (200 micron pore diameter) and bovine serum albumin (Sigma Chem.) is added (final concentration = 1%). For long-term culture experiments sterile technique is practiced. All solutions are passed through a 0.2 micron filter before contacting tissue. All tools are sterilized by autoclave. Final steps in the dispersion are carried out in a laminar flow hood (NuAire 300) to maintain sterility. After the mincing step the tissue fragments are mildly agitated in 10 mil. Medium 199 with 10% canine serum and 50 units/ml. each of penicillin and streptomycin (Irvine). Tissue debris is strained with sterile nylon mesh and medium is added to bring the final volume to 10 ml. Aliquots (2.0 ml.) are transferred to 35 mm. diameter Falcon culture dishes (5 per atrium or sinus node).

The dishes are stored in a water-jacketed, humidified 5% CO_2 incubator (Forma 3158) at 37°. Aliquots (0.2 ml.) are aseptically removed from them and placed in a glass-bottomed chamber (0.5 ml.). In this chamber cells adhere to the collagen treated bottom so that they can be suffused with fresh media. Cells are views through an inverted microscope (Nikon Diaphot) resting upon a compressed gas suspension table (Micro-G) to suppress vibration. Cell density is typically 50 $\pm$ 25 cells per field of view at 400 power magnification.

Suitably constructed microelectrodes can remove a patch of cell membrane and record the passage of current through it. Both transmembrane potential and chemical composition of the solutions bathing the patch are controlled to determine what kinds of ion-channels are present. Micropipettes are pulled in a 2-stage process so that the final product has a 2.0 micron outside diameter tip with 0.5 micron diameter opening. It is again heated in a microforge to remove any jagged edges. The shaft of the pipette is insulated with a layer of Q-dope all the way to the tip.

Negative pressure in the pipette lumen draws a cell membrane tightly against the opening and the membrane bonds electrostatically to the exposed glass ring of the pipette tip. When the membrane bonds to the entire ring of glass, it creates a high resistance (giga-ohms). Thus, current passing through the pipette traverses the relatively lower resistance of the membrane patch. This current is both injected and recorded with an Axopatch Patch Clamp system.

The membrane patch is either attached to the cell membrane or removed, which is desirable when the ionic composition of the solution exposed to the (formerly) intracellular membrane surface must be controlled precisely. Trans-patch voltage gradient is set with the patch clamp amplifier. At certain levels of transmembrane potential voltage-sensitive ion-specific channels open and close rapidly and these appear as high frequency (depending on temperature and voltage) current spikes of constant amplitude (Figure 1 and 2). Current carriers are identified by changing the concentrations of Na^+, K^+, Ca^{++}, etc. to alter the driving forces on them during the channels' open phases.

To investigate characteristics of K^+ channels, K^+ is the only cation present and an impermeant anion is used (such as gluconate). For inside-out or cell-attached type patches, the bath containes 150 mM. K gluconate, 5 mM. HEPES, and the pipette contains the same plus 1 mM. Ca^{++}. With inside-out patches and the same K^+ concentration on both sides, the reversal potential is zero, i.e., no current seen at mv. In other inside-out or cell-attached type patches, the reversal potential is determined. In these cases various combinations of bath and pipette solution compositions (75 mM., 150 mM., or 300 mM. K gluconate) result in varying reversal potentials depending upon the direction of K^+ concentration gradient.

To detect the presence of Ca^{++} -activated K^{+} channels, the Ca^{++} concentration in the pipette of inside-out patches is varied (in different patches) between 0.5 and 1000 micromolar. The Ca^{++} concentration in the bath of outside-out patches is varied also between 0 and 2 mM. The Ca^{++} concentrations are buffered to insure accurate concentrations by using appropriate combinations of Ca^{++} and EDTA and measurement with a Ca^{++} -sensitive electrode (Orion).

Canine right atria are electrophysiologically mapped and arterially perfused with collangenase (0.1%). After the dispersed cells are transferred to culture dishes (5.0 ml.), individual cells and clusters of cells are observed in an inverted microscope. The nominal pacemaker cells and working muscle cells are visually identified and impaled with microelectrodes to record spontaneous electrical activity. The technique used to disperse nominal pacemaker cells reduces the working muscle cell population of the sinus node sample.

Project Title: Effects of Trichothecenes on Cerebral Blood Flow and Pulmonary Vascular Resistance.

The model described in the previous progress report was used to answer 2 questions this quarter. First, in 6 rats injected intravenously with T-2 toxin or Roridin A (up to 4.0 mg./kg.), no changes in cerebral blood flow were detected by the hydrogen clearance technique (see Figure 3). Second, in the same animals catheters advanced into the inferior vena cavae recorded no changes in venous pressure. This supports the hypothesis that trichothecenes do not cause pulmonary arteriolar constriction (pulmonary hypertension) as does endotoxin. Therefore, by elimination of other possibilities, we conclude that the major effect on the autonomic nervous system may be mediated by membrane effects or by interference at the myoneural junction. Further studies will be performed to determine whether

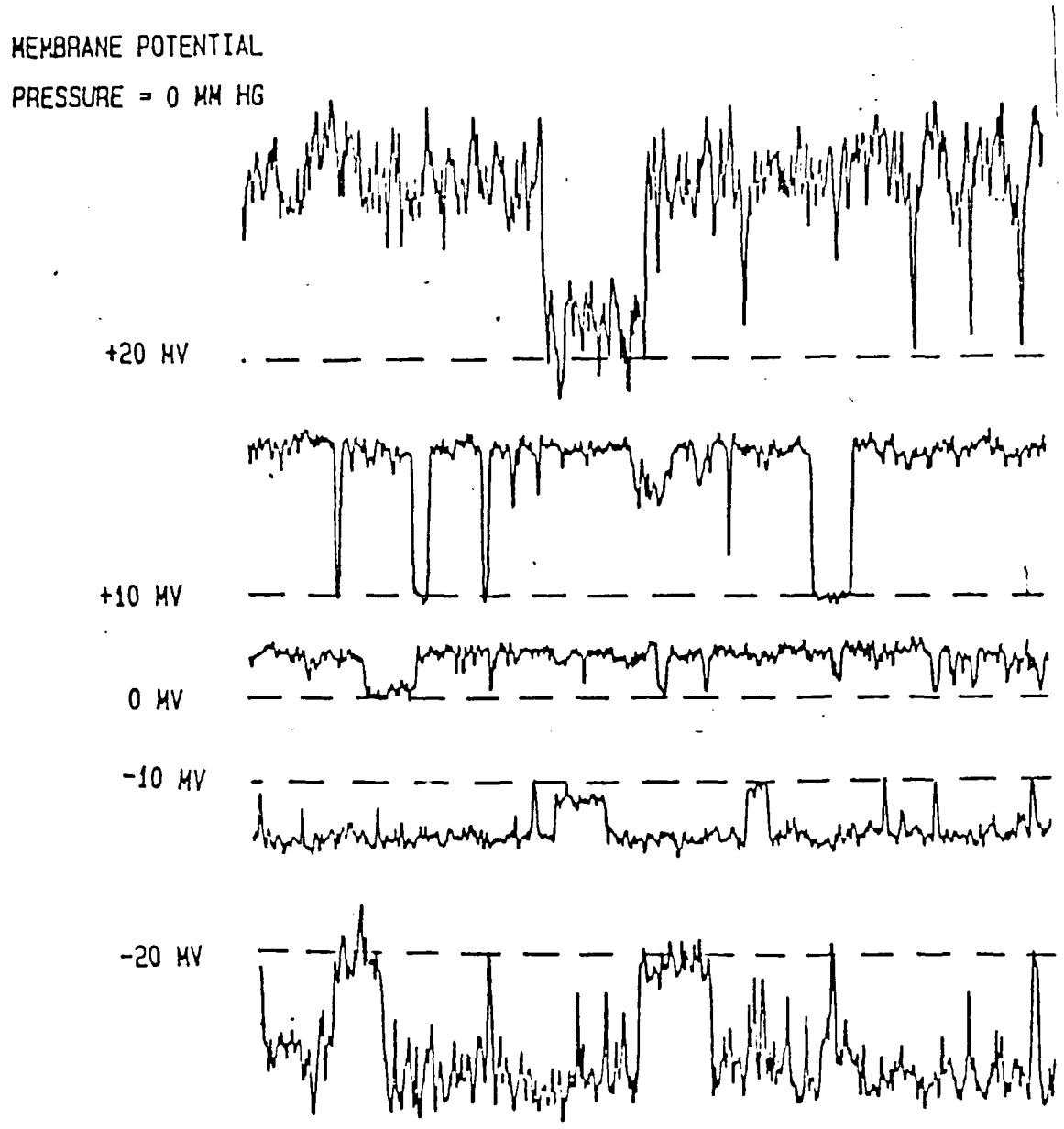

Figure 1. Currents recorded from an inside/out patch of pacemaker cell membrane are shown at 5 different holding potentials. For current calibration the unitary events of 0 mv. are 1.8 picoamperes in amplitude. Upward deflections are outward current; downward deflections are inward currents. Closed level = dashed line. Each record is 1.0 sec in length.

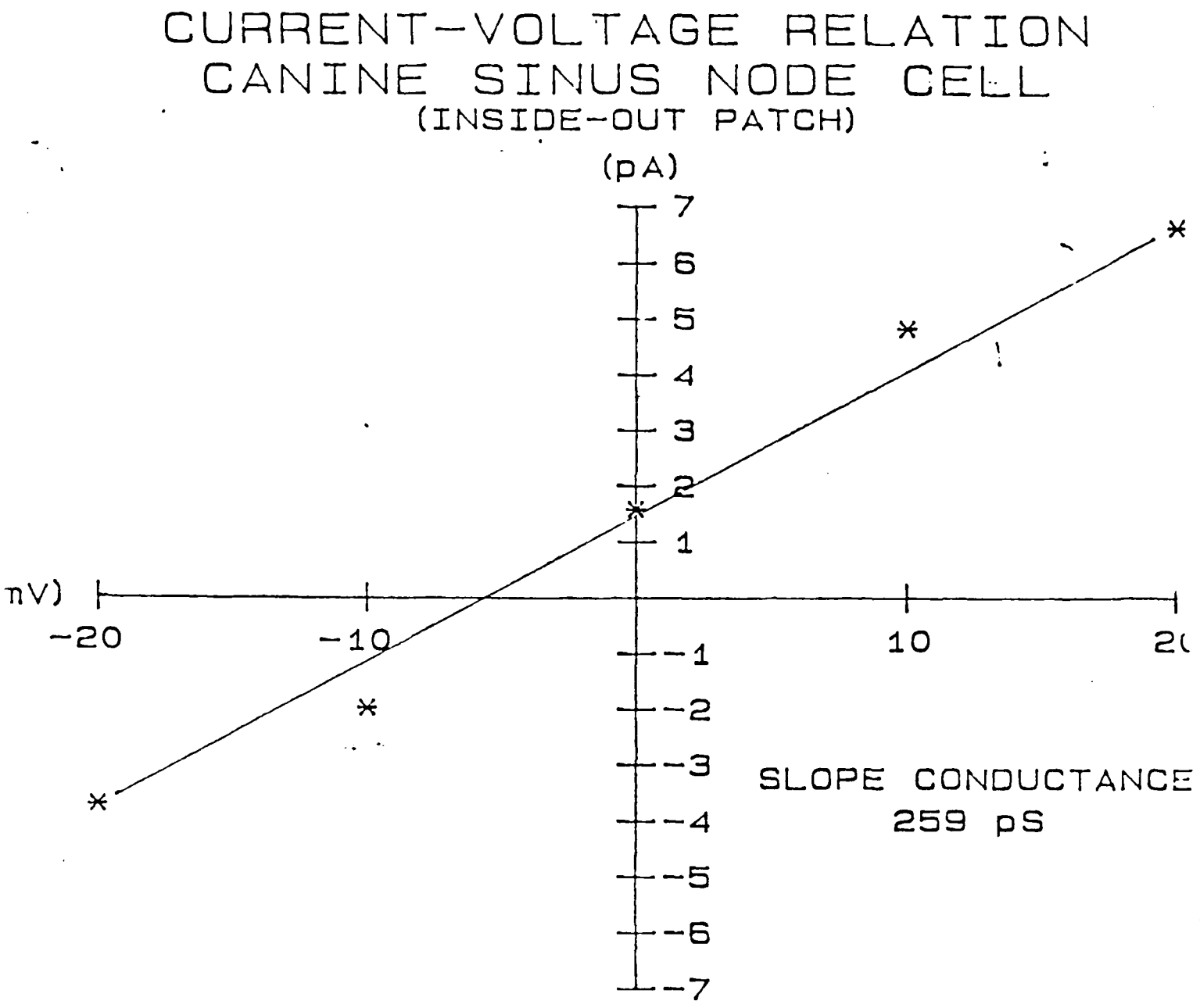

Figure 2. The current/voltage plot for data in Figure 2 is shown.

the effect is pre- or post-synaptic. For example, we will selectively block vascular adrenergic receptors to test for post-synaptic effects and membrane effects will be assessed by the patch clamp technique.

SUMMARY OF RESULTS

Single channel electrical activity has been characterized in canine atrial myocytes and pacemaker cells. We can, therefore, directly study changes in specific ion conductance pathways in the heart cell membrane. The following conductances have been recognized:

1. Ca^{++} -sensitive K^{+} conductance
2. Rectifying K^{+} conductance
3. Stretch-sensitive conductance (not voltage-sensitive)

One manuscript that was previously submitted became accepted for publication in Toxicon (Bubien, J.K. and Woods, W.T.,Jr., Direct and reflex effects of trichothecene mycotoxins). A manuscript was prepared for a book in preparation by V.R. Beasley (editor) to be entitled, Trichothecene Mycotoxicosis. Our chapter is to be entitled "Effects on the Cardiovascular System" [authors: J.K. Bubien, G. Lundeen (Univ. Ill.), C.B. Templeton (USAMRIID), and W.T. Woods, Jr.]. Experimentally the studies to test whether or not T-2 or Roridin A diminish cerebral blood flow were concluded. The "hydrogen clearance" technique was applied to microelectrodes inserted into each cerebral hemisphere of anesthetized rat brains to record changes in regional blood flow. No significant changes were observed to correlate with intravenous infusion of the mycotoxins from 0.5 to 2.0 mg. per kg.

To test the hypothesis that pulmonary arteriolar constriction contributes to the mycotoxicosis, we measured right atrial pressure with inferior vena caval catheters in anesthetized rats and dogs while T-2 and Roridin A were administered intravenously. Central venous pressure

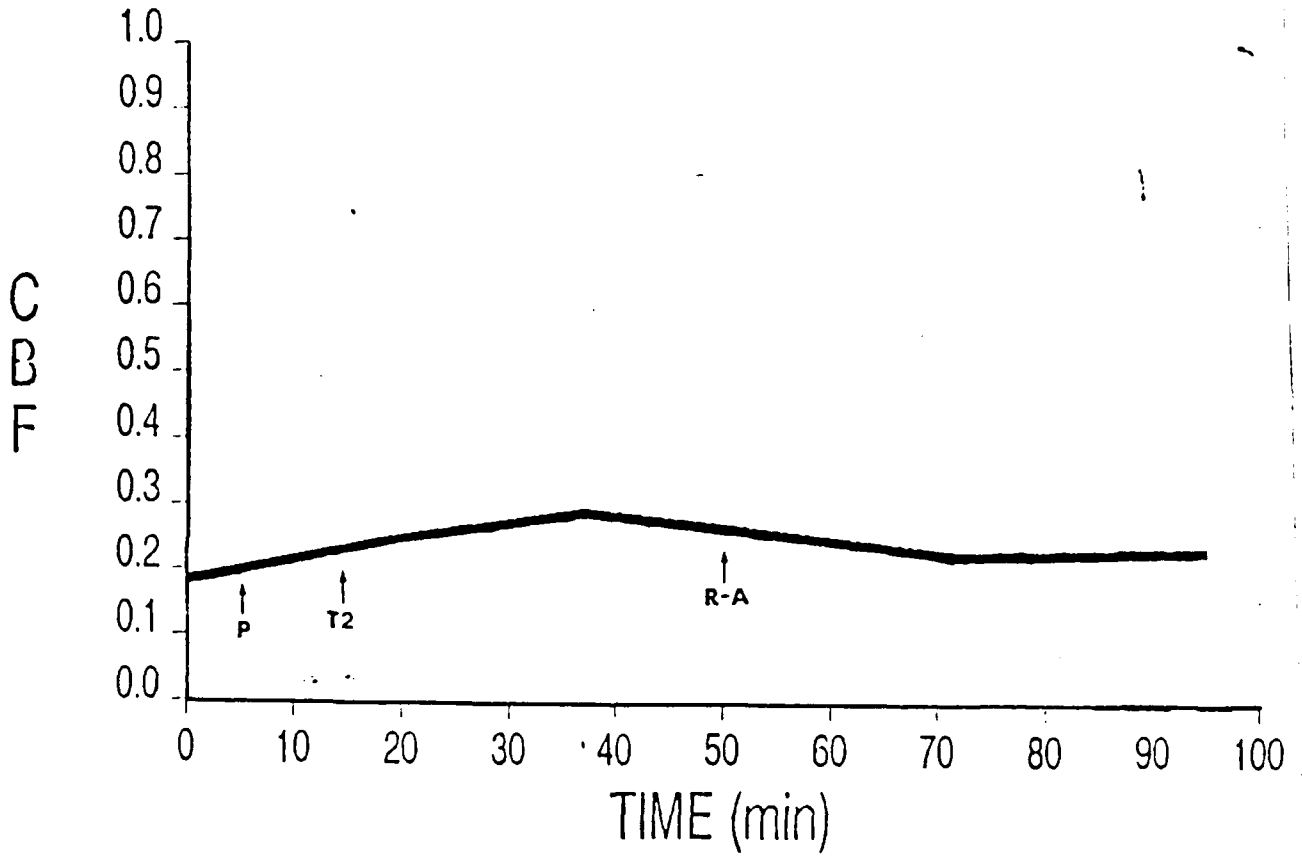

Figure 3. Cerebral blood flow (hydrogen clearance technique) remained unaffected by T-2 and Roridin-A. Likewise, central venous pressure remained unchanged for at lease 2 hours of exposure to toxins (4-8 mg./kg.) (data not show). P = propranolol (5 mg.); T-2 = T-2 toxin; R-A = Roridin-A; all intravenous injections.

remained constant throughout the 4 hr. observation period during which toxin concentrations were 0.5 to 2.5 mg. per kg.

SUMMARY

Experiments to determine the effects of trichothecenes on cardiovascular function were completed. In general, we conclude that the most prominent effects on the cardiovascular system are mediated via imbalance within the autonomic system caused by direct effects on autonomic neurons.

MECHANISMS OF ACTION OF OTHER TOXINS

1. Does the slow death (3-4 days in mice) component of microcystin operate on heart muscle or nerve cell membranes (i. e. to promote arrhythmias or dysautonomia)?

2. Does vital organ death from anatoxin A or S result from neuro-muscular, neural, or muscle cell inexcitability (like tetrodotoxin) or hyperexcitability (like batrachatoxin).?

3. Since binding of saxitoxin to cardiac cell membranes correlates poorly with electrophysiologic effects of saxitoxin, we propose to test the hypothesis that saxitoxin binds to Na+ channels of the conduction system rather than those of cardiac muscle.

Previous studies in this laboratory (13,27) revealed that tetrodotoxin (at a concentration adequate to produce atrial quiescence in the isolated canine heart) has only a brief negative effect on pacemaker firing rate. However, when Mg^{++} concentration was reduced the effect of tetrodotoxin became enhanced; it was no longer simply transient and its negative chronotropic effect became substantial (lowered firing rate by 50%) (Figure

4). The hypothesis that tetrodotoxin and Mg^{++} share a common mechanism to regulate transmembrane current in pacemaker cells is under study.

As described previously, we are directing our attention to cardiac effects of certain low molecular weight toxins that have known specific effects on excitable cells. TTX, for example, arrests activity of cardiac muscle cells but has no effect on cells in the cardiac conduction system which is responsible for generation and conduction of the cardiac electrical impulse. We have, therefore, performed experiments to test the hypothesis that cardiac function depends upon Na^+ channels that play different roles in different kinds of cardiac cells.

A poster was presented at the February 1987 meeting of the Biophysical Society in which preliminary studies were described. BTX binds to neural and muscle Na^+ channel receptors and holds them in the open configuration, in contradistinction to TTX which promotes the closed or blocked condition. In freshly isolated canine atrial cells we have observed that in the presence of BTX single channels carrying inward Na^+ current (the only charge carrier available) open repeatedly. Furthermore, when TTX was added, these unitary events ceased. Veratridine, which has the same action as BTX, was less potent by at least 100 fold as reported by other laboratories for nerve cells. We use the IPROC program of Sachs et al. modified for our LSI 1173 computer to analyse single channel data. BTX-induced channel characteristics are shown by Figures 5, 6, and 7.

RESULTS

Na^+ channels can be opened by exposure to BTX and veratridine in canine atrial myocytes. They can be blocked by TTX. By experimentally counter-posing these 2 effects, we will determine specific roles played by

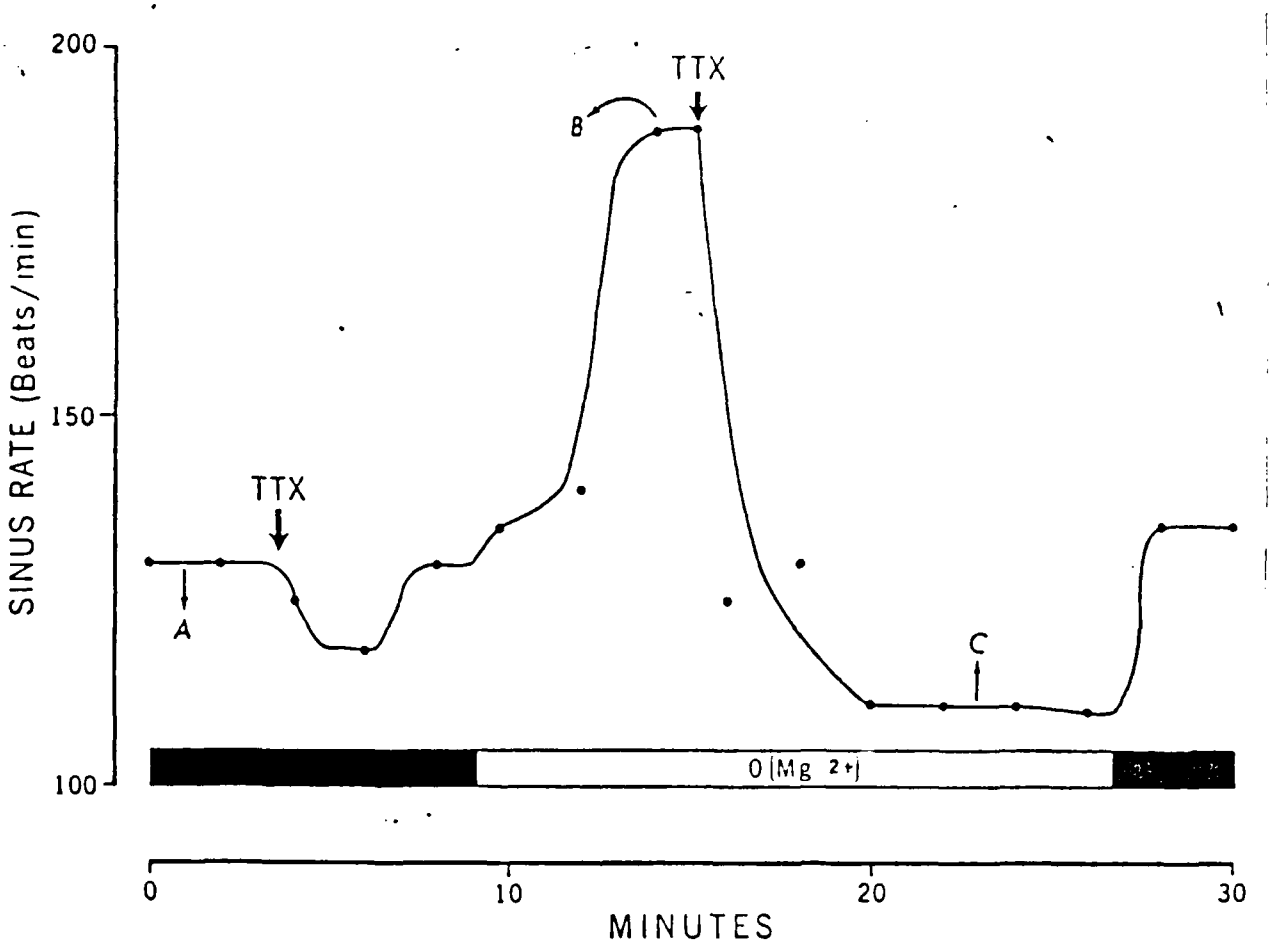

Figure 4. Sinus rate in this experiment remained (see point A) stable (130 bpm) until Mg^{++} was removed (unfilled horizontal bar) at which point it increased to 190 bpm (see point B). Tetrodotoxin (TTX 1.0 x 10^{-7} molar) added before Mg^{++} was removed (arrow) caused only a transient rate decrease, but during hypomagnesemia, TTX substantially lowered sinus rate (Point C).

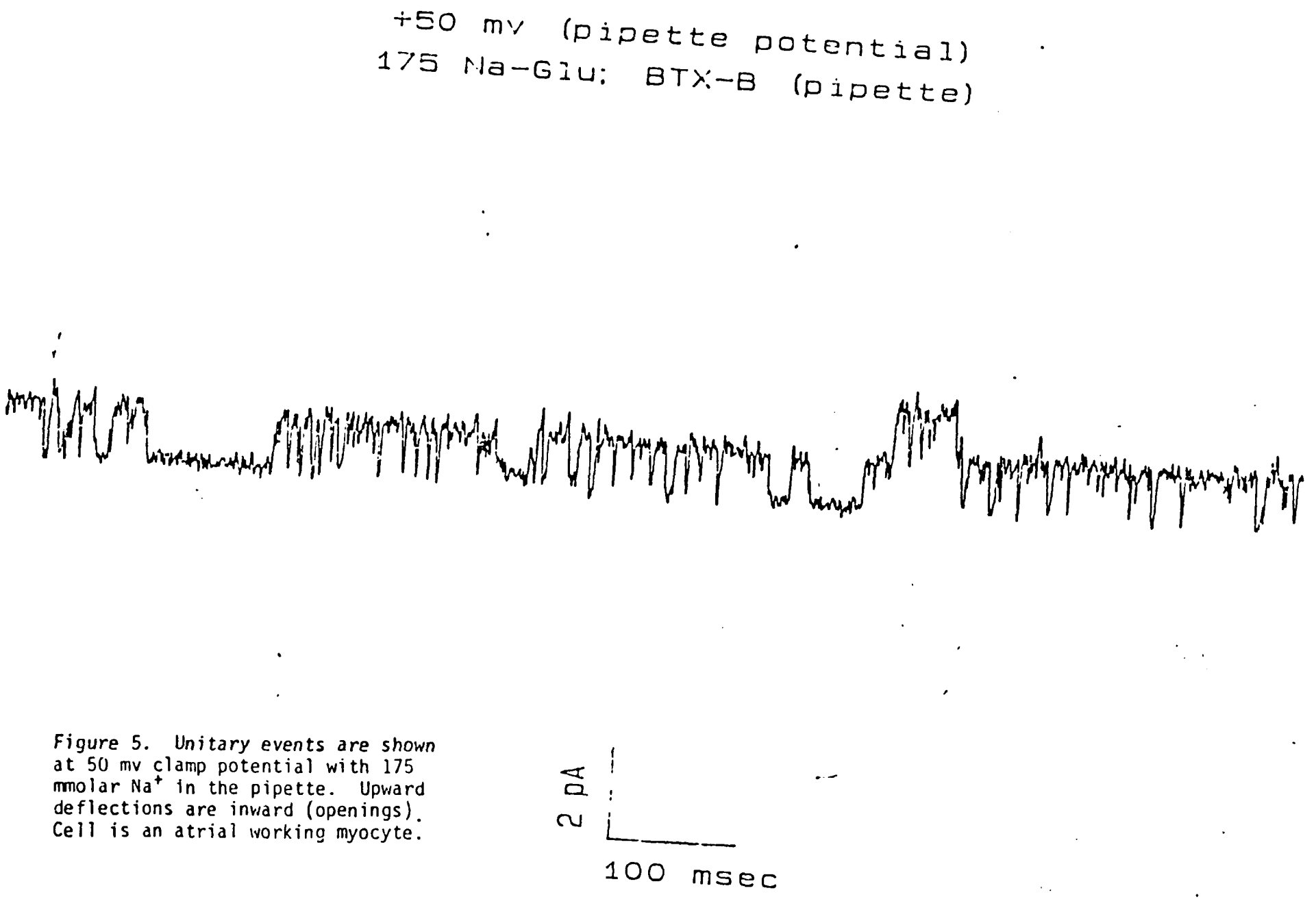

Figure 5. Unitary events are shown at 50 mv clamp potential with 175 mmolar Na^+ in the pipette. Upward deflections are inward (openings). Cell is an atrial working myocyte.

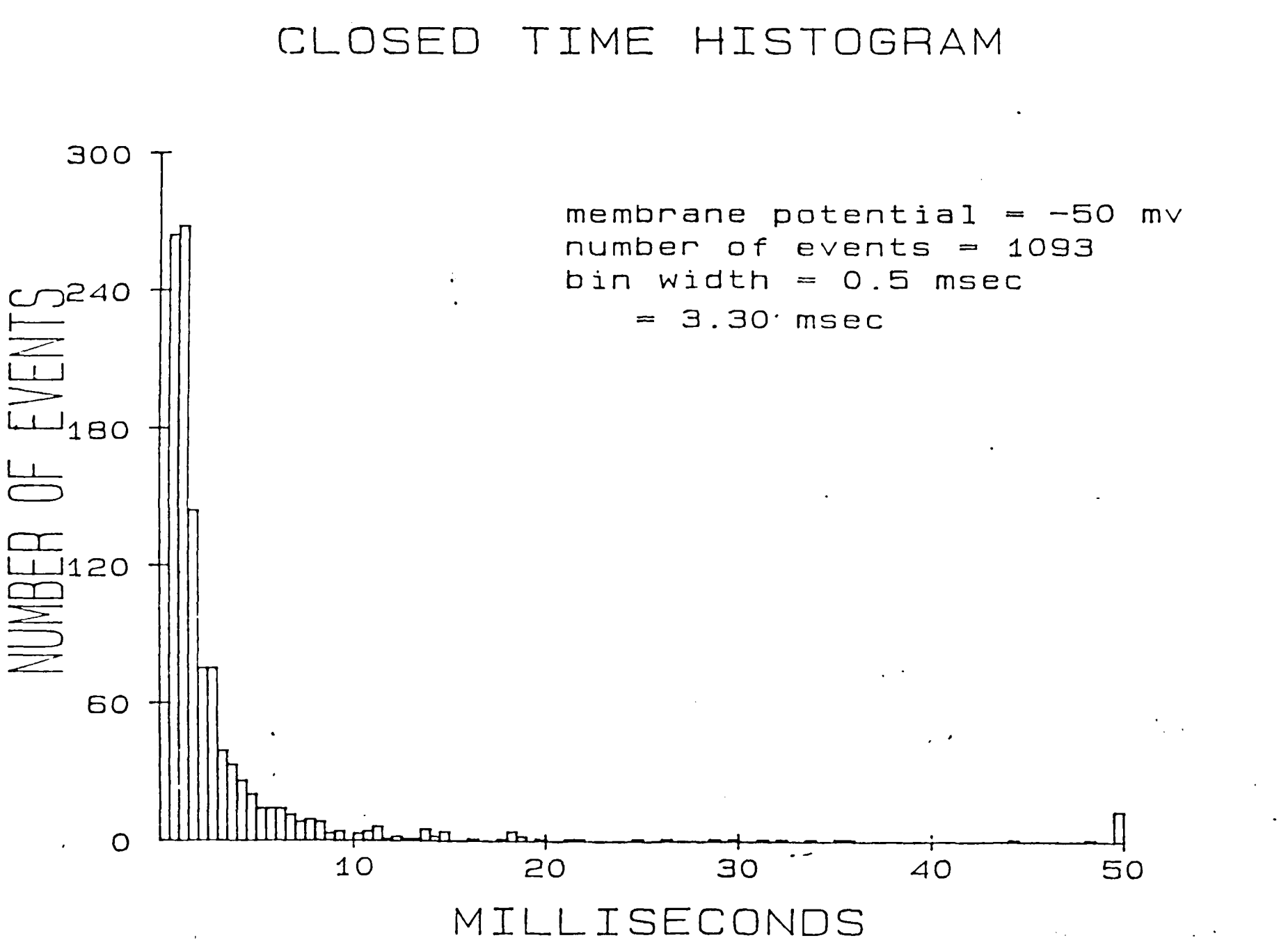

Figure 6. This histogram shows the distribution of closed times for the Na^+ channel. .The time constant was 3.3 msec.

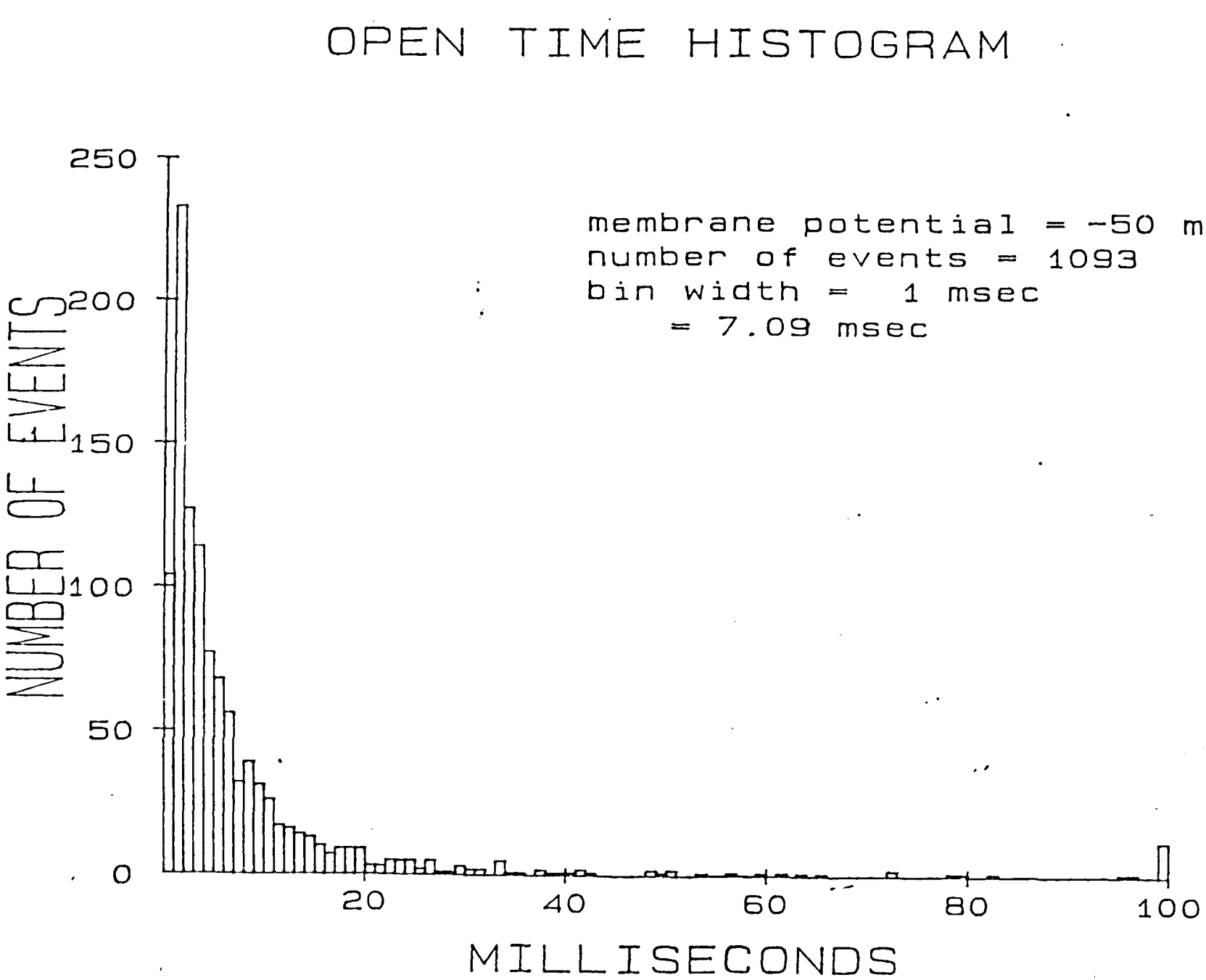

Figure 7. This histogram shows the distribution of closed times for the Na^+ channel. The time constant was 7.09 msec.

Na^+ channels in the cardiac muscle versus conduction cells and also we will investigate mechanisms by which these agents antagonize each other as well as saxitoxin and anemone-toxin which have related effects.

DISCUSSION AND CONCLUSIONS

Experiments revealed that single openings of Na^+ channels in cardiac cell membranes are difficult to study because of their voltage-and time-dependencies (they open only once and briefly for a single voltage step). Therefore, we tested effects of BTX and Veratridine (Na^+ channel openers) on single channels of cardiac muscle cells. The results indicated that they open channels that carry inward current and this can be blocked by TTX. They are being studied further as Na^+ channel agonists as well as TTX antagonists in cardiac cells.

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APPENDIX A

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ABSTRACTS

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